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MINIREVIEW

Reverse Genetics of Influenza Virus

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Reverse genetics of negative-sense RNA viruses, which enables one to generate virus entirely from cloned cDNA, has progressed rapidly over the past decade. However, despite the relative ease with which nonsegmented negative-sense RNA viruses can now be produced from plasmids, the ability to generate viruses with segmented genomes has lagged considerably, largely because of the inherent technical difficulties in providing all viral RNAs and proteins from cloned cDNA. A breakthrough in reverse genetics technology in the influenza virus field came in 1999, when we (Neumann *et al.,* 1999, *Proc. Natl. Acad. Sci. USA* 96, 9345–9350) and others (Fodor *et al.,* 1999, *J. Virol.* 73, 9679–9682) exploited a new approach to viral RNA production. In this review, we discuss the background for this advance, the systems that are now available for the generation of influenza viruses, and the implications of these developments for the future of virus research.

Influenza virus belongs to the family *Orthomyxoviridae* and contains a segmented RNA genome of negative polarity. In contrast to most other negative-sense RNA viruses, influenza virus replicates in the nucleus of infected cells (reviewed in Lamb and Krug, 1996). After receptor-mediated endocytosis and fusion of the viral and cellular membranes, the viral ribonucleoprotein (vRNP) complex is released into the cytoplasm. The vRNP complex, composed of the viral RNA (vRNA), nucleoprotein (NP), and three polymerase proteins (PB2, PB1, and PA), is transported to the nucleus where transcription and replication occur. The negative-sense vRNA (complementary to mRNA in its orientation) does not serve as a direct template for protein synthesis; rather, the vRNA encapsidated by NP must be transcribed into mRNA by the viral polymerase complex. During replication, the vRNA serves as a template for the synthesis of a full-length complementary RNA (cRNA), which in turn serves as a template for progeny vRNA synthesis. Thus, for influenza virus replication, the minimal functional unit is the vRNP complex. The generation of influenza A virus therefore requires eight functional RNP complexes that must be delivered into the cell nucleus.

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Attempts to generate influenza virus in the laboratory began in the 1980s, when replication-competent vRNPs were isolated from detergent-disrupted viruses (Plotch *et al.,* 1981) or infected cells (Beaton and Krug, 1986). These early studies demonstrated that vRNP complexes are sufficient for the replication of influenza virus RNA, but synthetic viral RNAs must be assembled into functional vRNP complexes to artificially generate influenza virus. This goal was partially reached in the late 1980s, when Parvin *et al.* (1989) and later Honda *et al.* (1990) reported the reconstitution of functional RNP complexes *in vitro.* Purified polymerase and NP proteins formed functional complexes that transcribed a synthetic RNA template and, more importantly, full-length viral RNA purified from virions. With the new-found ability to generate functional vRNP complexes, researchers directed their attention to techniques for introducing these (modified) vRNPs into virions.

In 1989, Palese and colleagues established the first system for modifying negative-sense RNA viruses (see Luytjes *et al.,* 1989), ushering in a new era of recombinant virus research using viruses containing vRNA derived solely from cloned cDNA (Fig. 1). Enami *et al.* (1990) assembled *in vitro* transcribed vRNA and purified NP and polymerase proteins into RNP complexes. Once these complexes were transfected into eukaryotic cells, helper virus infection provided the remaining vRNPs, resulting in a recombinant virus comprised of one of the vRNAs derived from cloned cDNA. However, in this system, the vast majority of progeny viruses are helper viruses, requiring strong selection systems based on antibody-

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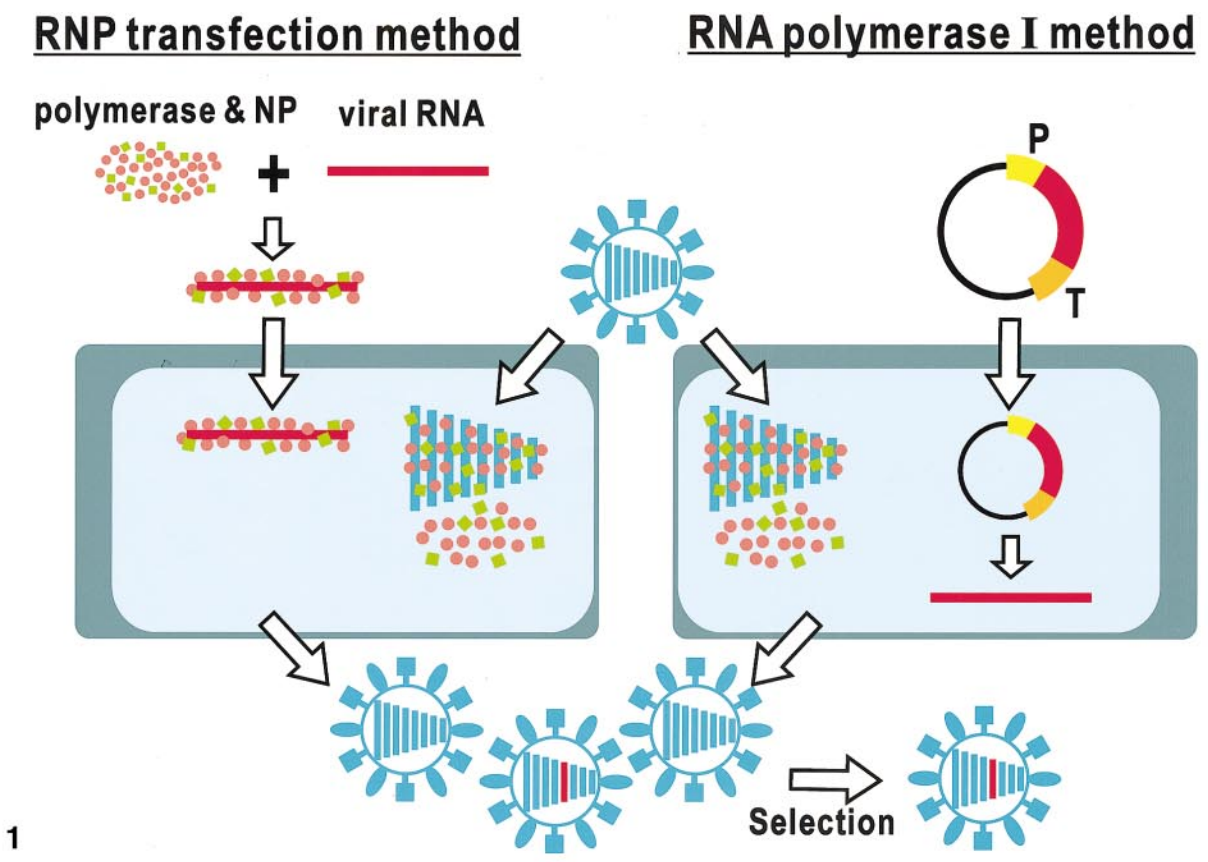
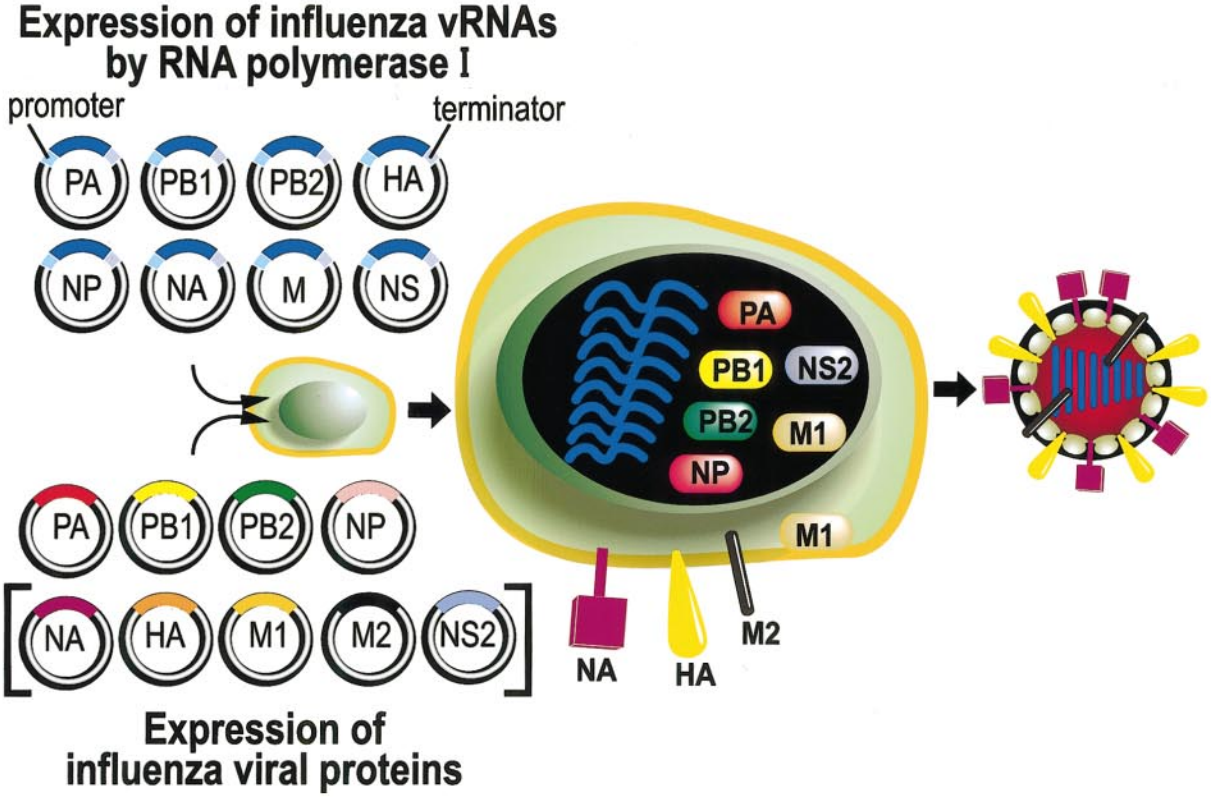

RNP transfection method
RNA polymerase I method
polymerase & NP
viral RNA
P
T
Selection

1


Expression of influenza vRNAs
by RNA polymerase I
promoter
terminator
PA
PB1
PB2
HA
NP
NA
M
NS
PA
PB1
NS2
PB2
M1
NP
M1
M2
NA
HA
PA
PB1
PB2
NP
NA
HA
M1
M2
NS2
Expression of
influenza viral proteins

2

mediated growth restriction (Barclay and Palese, 1995; Enami and Palese, 1991; Horimoto and Kawaoka, 1994; Rowley *et al.,* 1999), temperature sensitivity (Enami *et al.,* 1991; Li *et al.,* 1995; Yasuda *et al.,* 1994), host-range restriction (Enami *et al.,* 1990; Subbarao *et al.,* 1993), or drug resistance (Castrucci and Kawaoka, 1995). Although selection systems were eventually established for six of the eight influenza A viral segments [PB2 (Subbarao *et al.,* 1993), HA (Enami and Palese, 1991), NP (Li *et al.,* 1995), NA (Enami *et al.,* 1990), M (Castrucci and Kawaoka, 1995), and NS (Enami *et al.,* 1991)], and for the HA and NA segments of influenza B virus (Barclay and Palese, 1995; Rowley *et al.,* 1999), their application was technically demanding and the power of selection quite limited. Consequently, efforts for modifying the influenza virus genome using this experimental tactic were continued in only a few laboratories.

New impetus was lent to the reverse genetics of influenza virus when Hobom and colleagues, using RNA polymerase I for the intracellular synthesis of influenza virus RNA (Fig. 1), established an alternative system for the generation of influenza viral RNA (see Neumann *et al.,* 1994). RNA polymerase I is an abundant nuclear enzyme that transcribes ribosomal RNA (rRNA), which—like influenza vRNA—does not contain 5′-cap or 3′-poly(A) structures. Moreover, RNA polymerase I initiates and terminates transcription at defined promoter and terminator sequences that do not extend into the transcribed region. Hence, RNA polymerase I transcription yields transcripts that do not contain additional nucleotides at their 5′ or 3′ ends. Neumann *et al.* (1994) cloned a virus-like cDNA under the control of RNA polymerase I promoter and terminator sequences. After transfection, this group found that cellular RNA polymerase I synthesized an influenza virus-like RNA. Pleschka *et al.* (1996) employed this system to generate recombinant influenza virus by producing NA vRNP in the nucleus and infecting cells with helper virus to provide the remaining vRNP complexes, although selection systems were still required to isolate recombinant virus from the background of helper virus. The RNA polymerase I system is more convenient than the RNP transfection method because it circumvents the need for *in vitro* transcription, protein purification, and *in vitro* RNP assembly. However, with both of these helper virus-dependent systems, the efficiencies of virus generation were very low, precluding the generation of viruses with severe growth defects.

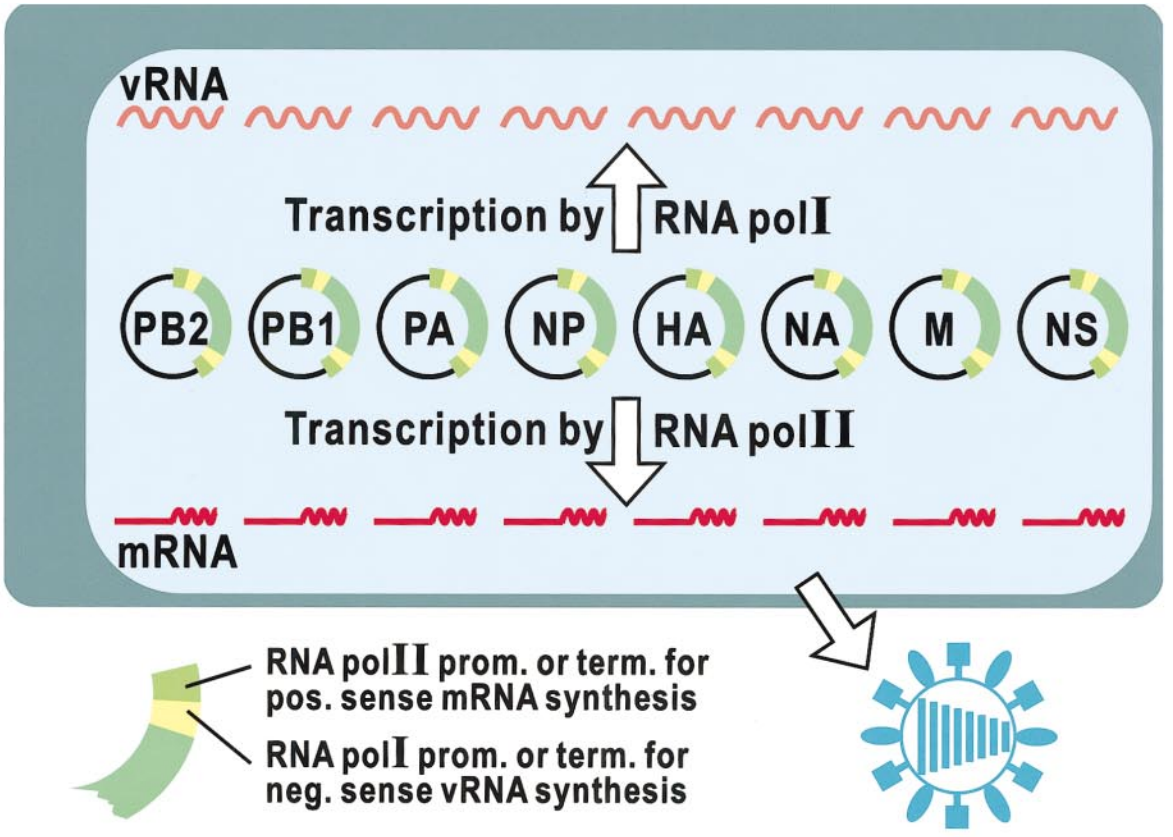
In 1994, Conzelmann and colleagues (see Schnell *et al.,* 1994) generated rabies virus (family *Rhabdoviridae*) from cloned cDNA for the first time for negative-stranded RNA viruses. A full-length cDNA construct encoding the antigenomic positive-sense cRNA was cloned under the control of the T7 RNA polymerase promoter and the hepatitis delta ribozyme. The resultant plasmid was transfected into eukaryotic cells that had been infected with recombinant vaccinia virus expressing T7 RNA polymerase. Cells were cotransfected with protein expression plasmids for the components of the viral transcription complex, all under control of the T7 RNA polymerase promoter. Similar approaches led to the production of all genera of the *Rhabdoviridae* and *Paramyxoviridae* families (Baron and Barret, 1997; Buchholz *et al.,* 1999; Clarke *et al.,* 2000; Collins *et al.,* 1995; Durbin *et al.,* 1997; Garcin *et al.,* 1995; Gassen *et al.,* 2000; He *et al.,* 1997; Hoffman and Banerjee, 1997; Jin *et al.,* 1998; Kato *et al.,* 1996; Lawson *et al.,* 1995; Peeters *et al.,* 1999; Radecke *et al.,* 1995; Romer-Oberdorfer *et al.,* 1999; Whelan *et al.,* 1995). These successes were soon translated into advances with segmented negative-sense RNA viruses.

In 1996, Bridgen and Elliott, using the approach outlined by Conzelmann's group (see Schnell *et al.,* 1994), produced a recombinant Bunyamwera virus, family *Bunyaviridae,* whose genome is composed of three segments of negative-sense RNA. Systems were thus in place for generating both nonsegmented and segmented negative-sense RNA viruses. A conspicuous exception was influenza virus, whose generation involves the added complexity of synthesis of eight (rather than three) viral RNAs, in addition to the polymerase and nucleoprotein from cloned cDNA.

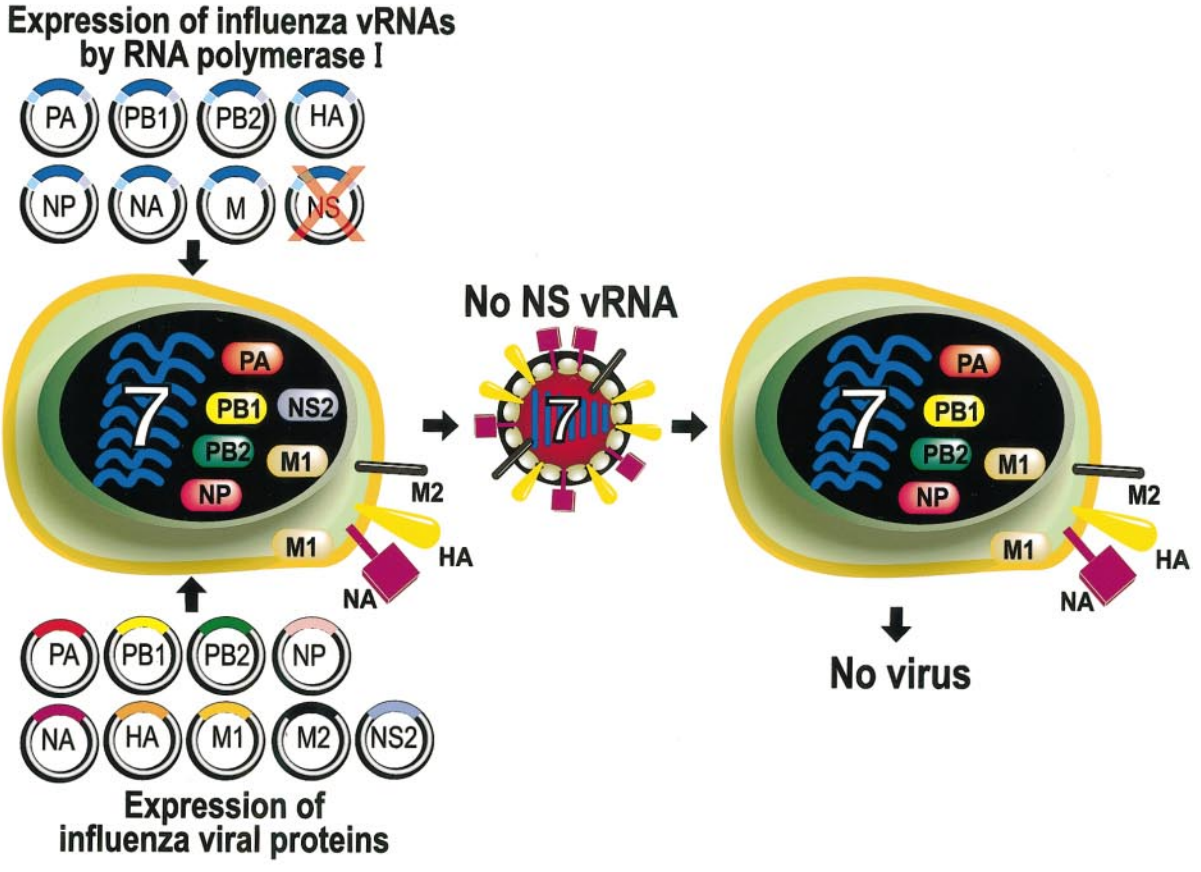
Technical obstacles to generating influenza virus entirely from cloned cDNA were overcome in 1999, when our research group cloned cDNAs encoding all eight segments of the A/WSN/33 (H1N1) virus between human RNA polymerase I promoter and mouse RNA polymerase I terminator sequences (Neumann *et al.,* 1999) (Fig. 2).

FIG. 1. Helper virus-dependent systems for the production of influenza virus possessing a single gene derived from cloned cDNA. In the ribonucleoprotein (RNP) transfection method, purified polymerase and nucleoprotein (NP) are mixed with *in vitro* synthesized vRNA to assemble functional RNP complexes. Cells are then transfected with the RNP complexes and infected with influenza helper virus (to provide the remaining seven RNPs). In the RNA polymerase I system, cells are transfected with a plasmid containing a cDNA that encodes one of the viral RNAs, flanked by RNA polymerase I promoter (P) and terminator (T) sequences. Transcription by cellular RNA polymerase I yields influenza vRNA that is replicated, transcribed, and packaged into progeny virus particles after infection with influenza helper virus. Both systems require stringent selection systems to select recombinant virus from the background of helper virus.

FIG. 2. "*De novo*" synthesis of influenza virus entirely from cloned cDNAs. Cells were transfected with eight plasmids that contain the RNA polymerase I promoter, a cDNA encoding one of the viral RNAs, and the RNA polymerase I terminator. Cotransfection of cells with four plasmids for the expression of viral proteins required for vRNA replication and transcription (PB1, PB2, PA, and NP) yields more than 1×10^8 infectious viruses per milliliter of supernatant from transfected cells. Cotransfection of cells with nine plasmids for the expression of all viral structural proteins permits production of virus particles lacking genes or possessing genes containing lethal mutations, thus allowing the generation of replication-incompetent virus-like particles for vaccine and gene delivery and functional analysis of viral proteins.


vRNA
Transcription by RNA polI
PB2
PB1
PA
NP
HA
NA
M
NS
Transcription by RNA polII
mRNA
RNA polII prom. or term. for pos. sense mRNA synthesis
RNA polI prom. or term. for neg. sense vRNA synthesis

3


Expression of influenza vRNAs by RNA polymerase I
PA
PB1
PB2
HA
NP
NA
M
NS
7
PA
PB1
NS2
PB2
M1
NP
M2
M1
HA
NA
No NS vRNA
7
7
PA
PB1
PB2
M1
NP
M2
M1
HA
NA
No virus
PA
PB1
PB2
NP
NA
HA
M1
M2
NS2
Expression of influenza viral proteins

4

After transfection of the resultant constructs in eukaryotic 293T (human embryonic kidney) cells, transcription by cellular RNA polymerase I yielded all eight vRNAs. In the initial experiments, cells were also cotransfected with plasmids expressing all nine structural proteins. Despite the introduction of 17 plasmids into cells (8 for the synthesis of RNA segments and 9 for protein expression), this approach yielded more than 1×10^7 infectious viruses per milliliter of supernatant from cells transfected with the plasmids. More recently, by improving the system's parameters, we have produced more than 1×10^8 infectious particles per milliliter of the supernatant, even with use of only four proteins (PA, PB1, PB2, and NP) for viral RNA transcription and replication. Although 12 plasmids are still required in our RNA polymerase I system for influenza A virus generation, it remains one of the most efficient systems yet reported (Pekosz *et al.,* 1999). We attribute this to the high transfection efficiency of 293T cells, leading to a pool of cells that receive the full complement of constructs required to initiate virus replication. Further, RNA polymerase I is abundantly expressed in growing cells, thus ensuring efficient replication of RNA polymerase I constructs. In addition to its high level of efficiency, the RNA polymerase I system is quite simple, requiring only DNA cloning and transfection techniques, which are well established in most virology laboratories. Fodor *et al.* (1999) also reported the generation of influenza A virus using a similar system; a hepatitis delta virus ribozyme was used instead of the RNA polymerase I terminator to produce vRNA possessing the authentic 3′ end sequence.

Most nonsegmented negative-sense RNA viruses have been generated from antigenomic, positive-sense cRNA (reviewed in Roberts and Rose, 1998). Either cRNA or vRNA was used to produce hPIV3 (Durbin *et al.,* 1997) and Sendai virus (Kato *et al.,* 1996); however, the latter template was less efficient in viral generation. For influenza virus production, negative-sense vRNA was superior to cRNA as a template for influenza virus production (Hoffmann and Webster, 2000; our unpublished data), possibly because with the nuclear RNA polymerase I system, it is unlikely that negative-sense vRNAs hybridize with positive-sense mRNAs in the cytoplasm, which is believed to affect the efficiency of virus generation in the T7 RNA polymerase system. Furthermore, stretches of uridine residues followed by hairpin structures, found in the genomes of nonsegmented negative-sense RNA viruses, may mimic the T7 RNA polymerase terminator structure, causing premature termination of transcription. By contrast, constraints that apply to the T7 RNA polymerase system, such as unwanted hybridization events or premature termination, do not impinge on the efficiency of virus generation in the RNA polymerase I system.

A modification of the RNA polymerase I system, designated RNA polymerase I/II system, was reported by Hoffmann *et al.* (2000) (Fig. 3). Briefly, a cDNA encoding a viral segment was cloned in the negative-sense orientation between RNA polymerase I promoter and terminator sequences, and this cassette was in turn cloned in the positive-sense orientation between an RNA polymerase II promoter (CMV early promoter) and a polyadenylation sequence. Transcription by RNA polymerase I yields negative-sense vRNA, whereas transcription by RNA polymerase II results in positive-sense mRNA synthesis. Thus, both vRNA and mRNA are generated from the same template, circumventing the need for protein expression constructs. Consequently, virus can be generated from 8 instead of 12 plasmids, reducing the number of plasmids required for virus generation. Therefore, this system may be useful for cell lines that otherwise cannot be transfected with a high level of efficiency. In the RNA polymerase I/II system, both protein expression and vRNA synthesis are achieved from the same template. Therefore, this system does not allow generation of virus-like particles lacking or containing lethal mutations in one or more viral segments. These limitations notwithstanding, both systems provide excellent tools for experimental mutagenesis directed at understanding influenza virus biology.

The RNA polymerase I system described above allows one to introduce any desired mutation into the genome of influenza virus. This capability opens the way to address such long-standing biologic issues as the nature of viral regulatory sequences, structure–function relationships, pathogenicity, and host cell tropism. Of particular importance is the identification of factors determining viral pathogenicity.

Perhaps the most intriguing potential application of reverse genetics systems for influenza virus generation is the creation of live-attenuated virus vaccines. Current inactivated vaccines effectively reduce the severity of

FIG. 3. The RNA polymerase I/II system for the generation of influenza virus. Cells are transfected with plasmids that contain a cDNA encoding a viral RNA flanked by RNA polymerase I promoter and terminator sequences in a negative-sense orientation. This cassette is then inserted between RNA polymerase II promoter and terminator sequences in a positive-sense orientation. Transfection of these plasmids results in influenza vRNA synthesis by cellular RNA polymerase I and in mRNA synthesis by cellular RNA polymerase II.

FIG. 4. Example of virus-like particles used to study the function of viral proteins. Cells are transfected with protein expression plasmids for all viral structural proteins and with RNA polymerase I constructs for the synthesis of seven viral RNAs, omitting the NS vRNA. The resulting particles, which contain only seven vRNAs, are used to infect fresh cells. Since proteins encoded by the NS segment are not expressed in subsequently infected cells, their contribution to the viral life cycle can be studied. In this study, RNP remained in the nucleus without the NS2 protein, indicating its contribution to RNP nuclear export (Neumann *et al.,* 2000a).

influenza-associated complications, but they do not prevent infection. A cold-adapted live-attenuated vaccine now in clinical trials (Maassab and Bryant, 1999) provides better protection than conventional inactivated vaccines in young children, but only comparable protection in adults (Boyce and Poland, 2000). Moreover, it contains only a limited number of amino acid replacements (Cox *et al.,* 1988; Herlocher *et al.,* 1996), suggesting a potential risk for emergence of a revertant virus upon use in a large population, even though the virus was phenotypically stable in clinical trials. Thus, there is room for improvement. The RNA polymerase I systems for virus generation are capable of producing a master strain of influenza virus with multiple attenuating mutations in the genes encoding internal proteins. In the near future, reverse genetics could be used to produce a high-yield reassortant virus that possesses the HA and NA from a currently circulating strain. Moreover, reverse genetics systems could be exploited in the production of inactivated vaccines, which currently are generated by conventional genetic reassortment procedures.

Influenza viruses may serve as potentially useful vectors for gene transfer into mammalian cells. Indeed, studies with helper virus-dependent reverse genetics systems have demonstrated that influenza virus can accommodate additional genetic material. For several short polypeptides, including the V3 loop of HIV-1 gp120 protein (Li *et al.,* 1993a), a highly conserved epitope from the ectodomain of HIV-1 gp41 (Muster *et al.,* 1994, 1995), a $CD8^+$ T-cell epitope from the circumsporozoite protein of *Plasmodium yoelli* (Li *et al.,* 1993b), and a B-cell epitope from the outer membrane protein F of *Pseudomonas aeruginosa* (Gilleland *et al.,* 1997), insertion in the antigenic sites of HA resulted in immune responses against the foreign epitope. For the expression of foreign full-length proteins, the gene of interest can be physically linked to one of the influenza virus genes. Protein expression can then be achieved by internal ribosomal entry sites (IRES) (Garcia-Sastre *et al.,* 1994) or the foot-and-mouth disease virus 2A protease (Percy *et al.,* 1994). In these approaches, the foreign genetic material is transcribed or translated as part of an influenza viral gene. Alternatively, foreign proteins can be expressed from an additional gene segment. Enami *et al.* (1991) rescued an artificial segment encoding wild-type NS1, but no NS2 protein, in a helper virus containing a *ts* mutation in NS1. Thus, this virus requires nine gene segments for efficient replication at a nonpermissive temperature, demonstrating the viability of influenza viruses containing more than eight genomic segments. To maintain additional segments in the absence of any selective pressure, one could use mutant influenza virus promoters that result in overreplication and/or overtranscription of the target gene (Neumann and Hobom, 1995), an approach explored by Zhou *et al.* (1998) for the generation of recombinant influenza virus expressing the E2 glycoprotein of classical swine fever virus.

Safe and efficient delivery of foreign genetic material into cells could be achieved by virus-like particles (VLPs), i.e., particles that do not contain the full complement of viral RNAs. The expression of all viral (structural) proteins and an influenza virus-like RNA encoding a reporter gene results in the efficient generation of VLPs (Mena *et al.,* 1996; Gomez-Puertas *et al.,* 1999; Neumann *et al.,* 2000b). The delivery of foreign genes could then be accomplished by transfection with protein expression constructs encoding all viral structural proteins and with RNA polymerase I constructs encoding the proteins required for replication and transcription (i.e., the polymerase and NP, together with the gene of interest). The resultant infectious particles could be used for a subsequent round of infection to deliver the foreign gene into target cells, where the polymerase and NP proteins would be expressed and drive replication and transcription of the target gene, resulting in its expression. Since the virus-like particles do not contain vRNAs encoding structural proteins, no progeny particles are generated after gene delivery, thus ensuring the safety of the delivery system. Furthermore, the availability of 15 HA and 9 NA subtypes would alleviate the risk of immunoresistance to vector-generated proteins, allowing the repeated administration of VLPs.

Reports are now beginning to emerge that illustrate the versatility of the new reverse genetics systems. Using the RNA polymerase I/II system, Hoffmann *et al.* (2000) regenerated an A/Teal/HK/W312/97 (H6N1) virus (isolated from a dead teal during the influenza virus outbreak in Hong Kong in 1997). Furthermore, they generated reassortants between the A/WSN/33 (H1N1) and the A/Teal/HK/W312/97 (H6N1) viruses and found that the introduction of the A/WSN/33 HA or NA genes into the Teal/HK/W312/97 background resulted in a significant reduction in virus titers. Thus, the approach outlined in this study may help researchers to elucidate the factors that determine viral pathogenicity (see Hatta *et al.,* in press).

Two other studies used the RNA polymerase I system to determine the function of influenza virus proteins. Neumann *et al.* (2000a) generated virus-like particles that entirely lacked or possessed mutations in the NS2 gene and examined the effect of these modifications on vRNP nuclear export (Fig. 4). This study confirmed, in the context of viral infection, a previous finding by O'Neill *et al.* (1998) that NS2 is critical for vRNP nuclear export, mediated by a nuclear export signal in the N-terminal region of NS2. Insights into the role of the M2 ion-channel protein were provided by Watanabe *et al.* (2001). Viruses were generated that lacked or contained mutations in the M2 transmembrane domain, indicating that influenza A viruses can undergo multiple cycles of replication without M2 ion-channel activity in cell culture.

However, viruses defective in M2 ion-channel activity did not efficiently replicate in mice, demonstrating that this activity is critical for the viral life cycle. Similar approaches could be employed to determine the functions of other influenza virus proteins or cellular events involving specific viral proteins.

RNA polymerase I-based systems for the generation of influenza A virus provide, for the first time, powerful tools for the modification of the influenza virus genome with only minimal technical difficulties. With wider application of this procedure, we can look forward to accelerated progress in understanding the influenza virus life cycle, the generation of live-attenuated vaccines, and the use of influenza A virus as vaccine and gene delivery vectors. Moreover, the RNA polymerase I system may prove useful in the "*de novo*" synthesis of other viruses in the *Orthomyxoviridae* family, including influenza B and C viruses and Thogotovirus, as well as segmented negative-sense RNA viruses of the *Bunyaviridae* or *Arenaviridae* families, which replicate in the cytoplasm of infected cells. Flick and Pettersson (2001) demonstrated the nuclear export of RNA polymerase I transcripts during synthesis of an Uukuniemi virus-like transcript (family *Bunyaviridae*). Thus, the RNA polymerase I system may provide an alternative method for generating nonsegmented negative-sense RNA viruses and could possibly result in more efficient production of these viruses than could be achieved with the conventional T7 RNA polymerase system.

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REFERENCES

Barclay, W. S., and Palese, P. (1995). Influenza B viruses with site-specific mutations introduced into the HA gene. *J. Virol.* **69,** 1275–1279.

Baron, M. D., and Barrett, T. (1997). Rescue of rinderpest virus from cloned cDNA. *J. Virol.* **71,** 1265–1271.

Beaton, A. R., and Krug, R. M. (1986). Transcription antitermination during influenza viral template RNA synthesis requires the nucleocapsid protein and the absence of a 5′ capped end. *Proc. Natl. Acad. Sci. USA* **83,** 6282–8286.

Boyce, T. G., and Poland, G. A. (2000). Promises and challenges of live-attenuated intranasal influenza vaccines across the age spectrum: A review. *Biomed. Pharmacother.* **54,** 210–218.

Bridgen, A., and Elliott, R. M. (1996). Rescue of a segmented negative-strand RNA virus entirely from cloned complementary DNAs. *Proc. Natl. Acad. Sci. USA* **93,** 15400–15404.

Buchholz, U. J., Finke, S., and Conzelmann, K. K. (1999). Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *J. Virol.* **73,** 251–259.

Castrucci, M. R., and Kawaoka, Y. (1995). Reverse genetics system for generation of an influenza A virus mutant containing a deletion of the carboxyl-terminal residue of M2 protein. *J. Virol.* **69,** 2725–2728.

Clarke, D. K., Sidhu, M. S., Johnson, J. E., and Udem, S. (2000). Rescue of mumps virus from cDNA. *J. Virol.* **74,** 4831–4838.

Collins, P. L., Hill, M. G., Camargo, E., Grosfeld, H., Chanock, R. M., and Murphy, B. R. (1995). Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5′ proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. *Proc. Natl. Acad. Sci. USA* **92,** 11563–11571.

Cox, N. J., Kitame, F., Kendal, A. P., Maassab, H. F., and Naeve C. (1988). Identification of sequence changes in the cold-adapted, live attenuated influenza vaccine strain, A/Ann Arbor/6/60 (H2N2). *Virology* **167,** 554–567.

Durbin, A. P., Hall, S. L., Siew, J. W., Whitehead, S. S., Collins, P. L., and Murphy, B. R. (1997). Recovery of infectious human parainfluenza virus type 3 from cDNA. *Virology* **235,** 323–332.

Enami, M., Luytjes, W., Krystal, M., and Palese, P. (1990). Introduction of site-specific mutations into the genome of influenza virus. *Proc. Natl. Acad. Sci. USA* **87,** 3802–3805.

Enami, M., and Palese, P. (1991). High-efficiency formation of influenza virus transfectants. *J. Virol.* **65,** 2711–2713.

Enami, M., Sharma, G., Benham, C., and Palese, P. (1991). An influenza virus containing nine different RNA segments. *Virology* **185,** 291–298.

Flick, R., and Pettersson, R. F. (2001). Reverse genetics system for Uukuniemi virus (*Bunyaviridae*): RNA polymerase I-catalyzed expression of chimeric viral RNAs. *J. Virol.* **75,** 1643–1655.

Fodor, E., Devenish, L., Engelhardt, O. G., Palese, P., Brownlee, G. G., and Garcia-Sastre, A. (1999). Resuce of influenza A virus from recombinant DNA. *J. Virol.* **73,** 9679–9682.

Garcia-Sastre, A., Muster, T., Barclay, W. D., Percy, N., and Palese, P. (1994). Use of a mammalian internal ribosomal entry site element for expression of a foreign protein by a transfectant influenza virus. *J. Virol.* **68,** 6254–6261.

Garcin, D., Pelet, T., Calain, P., Roux, L., Curran, J., and Kolakofsky, D. (1995). A highly recombinogenic system for the recovery of infectious Sendai paramyxovirus from cDNA: Generation of a novel copy-back nondefective interfering virus. *EMBO J.* **14,** 6087–6094.

Gassen, U., Collins, F. M., Duprex, W. P., and Rima, B. K. (2000). Establishment of a rescue system for canine distemper virus. *J. Virol.* **74,** 10737–10744.

Gilleland, H. E., Jr., Gilleland, L. B., Staczek, J., Harty, R. N., Garcia-Sastre, A., Engelhardt, O. G., and Palese, P. (1997). Chimeric influenza viruses incorporating epitopes of outer membrane protein F as a vaccine against pulmonary infection with *Pseudomonas aeruginosa*. *Behring. Inst. Mitt.* **98,** 291–301.

Gomez-Puertas P., Mena, I., Castillo M., Vivo, A., Perez-Pastrana, E., and Portela, A. (1999). Efficient formation of influenza virus-like particles: Dependence on the expression levels of viral proteins. *J. Gen. Virol.* **80,** 1635–1645.

Hatta, M., Gao, P., Halfmann, P., and Kawaoka, Y. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses, *Science,* in press.

He, B., Paterson, R. G., Ward, C. D., and Lamb, R. A. (1997). Recovery of infectious SV5 from cloned DNA and expression of a foreign gene. *Virology* **237,** 249–260.

Herlocher, M. L., Clavo, A. C., and Maassab, H. F. (1996). Sequence comparison of A/AA/6/60 influenza viruses: Mutations which may contribute to attenuation. *Virus Res.* **42,** 11–25.

Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G., and Webster, R. G. (2000). A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc. Natl. Acad. Sci. USA* **97,** 6108–6113.

Hoffmann, E., and Webster, R. G. (2000). Unidirectional RNA polymerase I–polymerase II transcription system for the generation of influenza A virus from eight plasmids. *J. Gen. Virol.* **81,** 2843–2847.

Hoffman, M. A., and Banerjee, A. K. (1997). An infectious clone of human parainfluenza virus type 3. *J. Virol.* **71,** 4272–4277.

Honda, A., Mukaigawa, J., Yokoiyama, A., Kato, A., Ueda, S., Nagata, K., Krystal, M., Nayak, D. P., and Ishihama, A. (1990). Purification and molecular structure of RNA polymerase from influenza virus A/PR8. *J. Biochem.* **107,** 624–628.

Horimoto, T., and Kawaoka, Y. (1994). Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. *J. Virol.* **68,** 3120–3128.

Jin, H., Clarke, D., Zhou, H. Z., Cheng, X., Coelingh, K., Bryant, M., and Li, S. (1998). Recombinant human respiratory syncytial virus (RSV) from cDNA and construction of subgroup A and B chimeric RSV. *Virology* **251,** 206–214.

Kato, A., Sakai, Y., Shioda, T., Kondo, T., Nakanishi, M., and Nagai, Y. (1996). Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative or positive sense. *Genes Cells* **1,** 569–579.

Lamb, R. A., and Krug, R. M. (1996). *Orthomyxoviridae:* The viruses and their replication. *In* "Fields Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), pp. 1353–1395. Lippincott–Raven, Philadelphia.

Lawson, N. D., Stillman, E. A., Whitt, M. A., and Rose, J. K. (1995). Recombinant vesicular stomatitis viruses from DNA. *Proc. Natl. Acad. Sci. USA* **92,** 4477–4481.

Li, S., Polonis, V., Isobe, H., Zaghouani, H., Guinea, R., Moran, T., Bona, C., and Palese, P. (1993a). Chimeric influenza virus induces neutralizing antibodies and cytotoxic T cells against human immunodeficiency virus type 1. *J. Virol.* **67,** 6659–6666.

Li, S., Rodrigues, M., Rodriguez, D., Rodgriguez, J. R., Esteban, M., Palese, P., Nussenzweig, R. S., and Zavala, F. (1993b). Priming with recombinant influenza virus followed by administration of recombinant vaccinia virus induces $CD8^+$ T-cell-mediated protective immunity against malaria. *Proc. Natl. Acad. Sci.* **90,** 5214–5218.

Li, S., Xu, M., and Coelingh, K. (1995). Electroporation of influenza virus ribonucleoprotein complexes for rescue of the nucleoprotein and matrix genes. *Virus Res.* **37,** 153–161.

Luytjes, W., Krystal, M., Enami, M., Pavin, J. D., and Palese, P. (1989). Amplification, expression and packaging of a foreign gene by influenza virus. *Cell* **58,** 1107–1113.

Maassab, H. F., and Bryant, M. L. (1999). The development of live attenuated cold-adapted influenza virus vaccine for humans. *Rev. Med. Virol.* **9,** 237–244.

Mena, I., Vivo, A., Perez, E., and Portela, A. (1996). Rescue of a synthetic chloramphenicol acetyltransferase RNA into influenza virus-like particles obtained from recombinant plasmids. *J. Virol.* **70,** 5016–5024.

Muster, T., Guinea, R., Trkola, A., Purtscher, M., Klima, A., Steindl, F., Palese, P., and Katinger, H. (1994). Cross-neutralizing activity against divergent human immunodeficiency virus type 1 isolates induced by the gp41 sequence ELDKWAS. *J. Virol.* **68,** 4031–4034.

Muster, T., Ferko, B., Klima, A., Purtscher, M., Trkola, A., Schulz, P., Grassauer, A., Engelhardt, O. G., Garcia-Sastre, A., Palese, P., and Katinger, H. (1995). Mucosal model of immunization against human immunodeficiency virus type 1 with a chimeric influenza virus. *J. Virol.* **69,** 6678–6686.

Neumann, G., and Hobom, G. (1995). Mutational analysis of influenza virus promoter elements *in vivo*. *J. Gen. Virol.* **76,** 1709–1717.

Neumann, G., Zobel, A., and Hobom, G. (1994). RNA polymerase I-mediated expression of influenza viral RNA molecules. *Virology* **202,** 477–479.

Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D., Donis, R., Hoffmann, E., Hobom, G., and Kawaoka, Y. (1999). Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl. Acad. Sci.* **96,** 9345–9350.

Neumann, G., Hughes, M. T., and Kawaoka, Y. (2000a). Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. *EMBO J.* **19,** 6751–6758.

Neumann, G., Watanabe, T., and Kawaoka, Y. (2000b). Plasmid-driven formation of influenza virus-like particles. *J. Virol.* **74,** 547–551.

O'Neill, R. E., Talon, J., and Palese, P. (1998). The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. *EMBO J.* **17,** 288–296.

Parvin, J. D., Palese, P., Honda, A., Ishihama, A., and Krystal, M. (1989). Promoter analysis of influenza virus RNA polymerase. *J. Virol.* **68,** 5142–5152.

Peeters, B. P., de Leeuw, O. S., Koch, G., and Gielkens, A. L. (1999). Rescue of Newcastle disease virus from cloned cDNA: Evidence that cleavability of the fusion protein is a major determinant for virulence. *J. Virol.* **73,** 5001–5009.

Pekosz, A., He, B., and Lamb, R. A. (1999). Reverse genetic of negative-strand RNA viruses: Closing the circle. *Proc. Natl. Acad. Sci.* **96,** 8804–8806.

Percy, N., Barclay, W. S., Garcia-Sastre, A., and Palese, P. (1994). Expression of a foreign protein by influenza A virus. *J. Virol.* **68,** 4486–4492.

Pleschka, S., Jaskunas, R., Engelhardt, O. G., Zurcher, T., Palese, P., and Garcia-Sastre, A. (1996). A plasmid-based reverse genetics system for influenza A virus. *J. Virol.* **70,** 4188–4192.

Plotch, S. J., Bouloy, M., Ulmanen, I., and Krug, R. M. (1981). A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. *Cell* **23,** 847–858.

Radecke, F., Spielhofer, P., Schneider, H., Kaelin, K., Huber, M., Dotsch, C., Christiansen, G., and Billeter, M. A. (1995). Rescue of measles virus from cloned DNA. *EMBO J.* **14,** 5773–5784.

Roberts, A., and Rose, J. K. (1998). Recovery of negative-strand RNA viruses from plasmid DNAs: A positive approach revatilizes a negative field. *Virology* **247,** 1–6.

Romer-Oberdorfer, A., Mundt, E., Mebatsion, T., Buchholz, U. J., and Mettenleiter, T. C. (1999). Generation of recombinant lentogenic Newcastle disease virus from cDNA. *J. Gen. Virol.* **80,** 2987–2995.

Rowley, K. V., Harvey, R., and Barclay, W. S. (1999). Isolation and characterization of a transfectant influenza B virus altered in RNA segment 6. *J. Gen. Virol.* **80,** 2353–2359.

Schnell, M. J., Mebatsion, T., and Conzelmann, K. K. (1994). Infectious rabies viruses from cloned cDNA. *EMBO J.* **13,** 4195–4203.

Subbarao, E. K., Kawaoka, Y., and Murphy, B. R. (1993). Rescue of an influenza A virus wild-type PB2 gene and a mutant derivative bearing a site-specific temperature-sensitive and attenuating mutation. *J. Virol.* **67,** 7223–7228.

Watanabe, T., Watanabe, S., Ito, H., Kida, H., and Kawaoka, Y. (2001). Influenza A virus can undergo multiple cycles of replication without M2 ion channel activity. *J. Virol.* **75,** 5656–5662.

Whelan, S. P., Ball, L. A., Barr, J. N., and Wertz, G. T. (1995). Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. *Proc. Natl. Acad. Sci. USA* **92,** 8388–8392.

Yasuda, J., Bucher, D. J., and Ishihama, A. (1994). Growth control of influenza A virus by M1 protein: Analysis of transfectant viruses carrying the chimeric M gene. *J. Virol.* **68,** 8141–8146.

Zhou, Y., Koenig, M., Hobom, G., and Neumeier, E. (1998). Membrane-anchored incorporation of a foreign protein in recombinant influenza virions. *Virology* **246,** 83–94.